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Microsatellite Variation and Differentiation in North Atlantic Eels

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Abstract

We screened 11 populations of American, European, and Icelandic eels (Anguillidae) for allelic variation and genetic divergence at six polymorphic microsatellite loci. Within either of the two recognized *Anguilla* species in the North Atlantic (*rostrata* in the Americas, *anguilla* in Europe), population genetic structure was statistically significant but weak; fully 95% of the total genetic variation was present within geographic locales rather than distributed among them. The two *Anguilla* species also overlap greatly in allelic frequencies, so the available data proved ineffective for addressing hypotheses about the possible hybrid origins of some Icelandic eels. The overlapping microsatellite profiles contrast with nearly diagnostic species differences documented previously in allozymes and mtDNA. This and similar empirical findings in several other species support theoretical concerns that homoplasy (convergent evolution) in allelic states can compromise the utility of rapidly mutating microsatellite loci for certain types of microevolutionary questions regarding gene flow and species differences.

Anguillid eels of the North Atlantic traditionally have been divided into two taxonomic species: *Anguilla rostrata* (American eels) from the Western Hemisphere, and *A. anguilla* (European eels) from Europe, Iceland, and north Africa. However, only one quasi-diagnostic morphological feature is known (Tesch 1977); individuals of *A. rostrata* exhibit 103–110 (mean 107.1) vertebrae (or myomeres in larvae), whereas the count in *A. anguilla* ranges from 110–119 (mean 114.7). Anguillid eels are catadromous, meaning that maturing individuals spend most of their lives in estuaries or freshwater but migrate to the ocean to reproduce. Based on the observed geographic distributions of newly hatched larvae, both American and European eels spawn in the tropical western Atlantic Ocean (the Sargasso Sea region). Thus both the morphological and distributional evidence raise questions as to whether these two "species" are indeed genetically distinct, and if so by how much, and what might account for their reproductive isolation.

In recent years, molecular genetic data have been gathered to address these and related issues [review in Avise (2003)]. More than a dozen allozyme loci were surveyed first [Williams and Koehn (1984) and references therein]. One of these proved to be nearly diagnostic for the two taxonomic species, with allele *Mdh-2*^{a} present in a frequency of about 0.96 in all American eel populations, but only 0.10 in European samples. Allele frequencies at the other allozyme loci differed little or not at all between *A. rostrata* and *A. anguilla*. Later analyses of mitochondrial DNA (mtDNA) restriction sites (Avise et al. 1986) uncovered the first definitively diagnostic genetic characters for the two species: all specimens grouped into two continent-specific mtDNA clades separated by about 3% net sequence divergence (after correction for mean within-taxon variation). These matrilineal distinctions between American and European eels subsequently were confirmed by direct mtDNA sequence analyses [see references in Avise (2003)].

Another long-standing question stemming from the eels' catadromous life history is whether conspecific populations from different locales within a continent have come from a panmictic spawn. Available allozyme data barely dispute this possibility; apart from mild clinal variation at a few loci, sample allele frequencies otherwise were indistinguishable across locales. Williams et al. (1984) provisionally attributed the clines to spatial variation in intra-generational selection pressures rather than departures from random mating within a generation. Recently, in assays of nuclear microsatellite loci, Wirth and Bernatchez (2001) reported statistically significant (albeit low) levels of geographic population structure in *A. anguilla* sampled across this species' vast European range, a result that they attributed to nonrandom mating and restricted gene flow.

Finally, questions have arisen about the genetic status of Icelandic eels. Although generally considered *A. anguilla*, populations in Iceland have a frequency distribution of vertebral counts that is shifted somewhat toward *A. rostrata* and slightly lower in mean than populations from continental Europe. This observation, coupled with nonrandom associations between vertebral counts and allozyme and

Table 1. Geographic population samples employed

Species	Population	Location (state or country)	No. of specimens
A. rostrata	Pamlico River	North Carolina	13
	Satilla River	Georgia	16
	Long Island	New York	26
	Penobscot River	Maine	13
A. anguilla	River Dee	England	14
	River Shannon	Ireland	14
	Aarhus	Denmark	16
	Reykholar	Iceland	48
	Oxnalaekur	Iceland	68
	Stokkseryi	Iceland	61
	Villingholtvatn	Iceland	26

mitochondrial genetic markers (Avise et al. 1990; Williams et al. 1984), raised the possibility that some Icelandic eels may be of mixed (hybrid) ancestry between *A. anguilla* and *A. rostrata*. Additional diagnostic markers from the nuclear genome are needed to critically test this hypothesis.

Here we compare multiple population samples of both *A. rostrata* and *A. anguilla* with respect to microsatellite markers. These data could shed further light on genetic relationships between these taxa, and perhaps also help to address several of the other questions raised here.

Materials and Methods

The nuclear DNA samples employed (Table 1) were stored at −20°C for more than a decade. They had been extracted from eels collected in earlier mtDNA surveys of American, European, and Icelandic populations (Avise et al. 1986, 1990).

Six microsatellite loci developed by Wirth and Bernatchez (2001) were amplified in the *Anguilla* samples with fluorescently labeled primers. Polymerase chain reactions (PCRs) of 10 μl consisted of 1 μl DNA, 1 μM each of forward and reverse primers, 7.5 μM of each dNTP (Roche), 5 U *Taq* DNA polymerase (Promega), and $MgCl_2$ concentrations as shown in Table 2, in 1× $MgCl_2$-free PCR buffer (Promega). Cycling conditions were a 5 min denaturation at 95°C, 30 cycles of 1 min at 95°C, 1 min at annealing temperature (shown in Table 2), and 1 min elongation at 72°C, followed by an 8 min final elongation at 72°C. For fluorescent DNA fragment analysis, the reactions were pooled and electrophoresed on polyacrylamide gels using an ABI 377 automated DNA sequencer with a 500 size standard (Applied Biosystems). Fragment data were extracted with GeneScan and analyzed using Genotyper (Applied Biosystems).

The genotypic data were entered into Arlequin (Schneider et al. 2000) to estimate genetic distance values and conduct Hardy–Weinberg tests. Values of G_{ST} (a measure of population differentiation for multiallelic loci; Nei and Kumar 2000) and their standard errors from 1,000 bootstrap repetitions were calculated using Poptree (Takezaki 1998). Another genetic distance measure, D_a (Nei et al. 1983), calculated using Dispan (Ohta 1993), served as the basis for generating a neighbor-joining (NJ) tree (Saitou and Nei 1987) to show relationships among population samples. The tree was midpoint rooted, and levels of confidence in tree nodes were calculated from 1,000 bootstrap replications.

Table 2. PCR conditions for the microsatellite assays

Microsatellite locus	$MgCl_2$ concentration (mM)	Annealing temperature (°C)
Aro121	2.25	60
Ang114	2.00	62
Aro095	1.75	50
Aro063	2.25	55
Ang151	1.75	60
Ang101	2.50	60

Results

Species' allelic frequencies at the six amplified loci are shown in Figure 1. The average total number of alleles per locus was 41 (range 30–57) and the mean observed heterozygosity per population was 0.82 (range 0.79–0.86), as shown in Table 2. Most of the loci conformed to Hardy–Weinberg expectations (HWE) at both the local-population and species levels. The only exceptions involved 16 of 78 tests (20%) that displayed a mild ($P < .05$) departure from HWE, but without a discernable pattern.

Table 3 summarizes the estimates of G_{ST} for population samples of *A. rostrata* and *A. anguilla* considered both separately and pooled. Values in all cases were low, but statistically significant. Figure 2 plots the observed ranges of genetic distances as measured by the D_a metric in various subsets of populations within and between the two nominal *Anguilla* taxa. The largest average pairwise distance was between American and European groups. Less distance was observed between pairwise comparisons of American and Icelandic groups. Figure 3 shows a NJ tree for the 11 surveyed eel populations, as generated from a matrix of these genetic distance values. When midpoint-rooted, this tree marginally separates all populations of American eels from those sampled in Iceland and Europe.

Discussion

Geographic Variation Among Conspecific Populations

Allelic variation at all six microsatellite loci was high, but only weakly partitioned among the geographic locales of each species. Thus, although both *A. anguilla and A. rostrata* show statistically significant population-genetic differentiation, the G_{ST} values were remarkably low considering the vast geographic ranges surveyed; fully 94–97% of the total genetic variation occurred within rather than among population samples. Furthermore, for such highly polymorphic loci, any apparent population structure based on

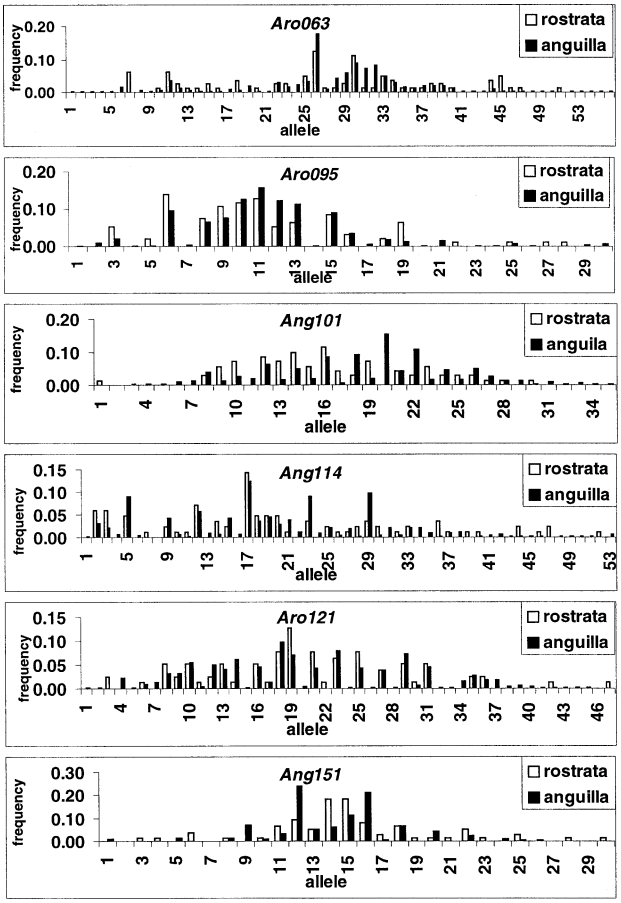

Figure 1. Allele frequency histograms at six microsatellite loci in the two recognized species of North Atlantic eels.

finite samples must be interpreted cautiously due to stochastic sampling errors in the allele frequency estimates and the strong likelihood that some rare alleles will be missed.

Our results are consistent with the low geographic differentiation reported in previous microsatellite assays of European eels (Wirth and Bernatchez 2001), and they extend such findings to the American species. Our study also generally agrees with the negligible population-genetic divergence reported in intraspecific allozyme surveys of North Atlantic eels, suggesting that any departures from apparent eel panmixia are modest at best (Williams and Koehn 1984). The relative paucity of spatial differentiation within either *A. rostrata* or *A. anguilla* on continent-wide scales is undoubtedly related to these animals' catadromous life history, which likely promotes a "concerted" genetic evolution of widely separated freshwater populations via the ongoing opportunity for interpopulational matings and range-wide juvenile dispersal in nearly every generation. These unusual peculiarities of eel natural history in the North Atlantic region appear to have greatly inhibited the accumulation of population-genetic differences over time, certainly in comparison to most other widely distributed species of freshwater and marine fishes that for reasons of geography and life history are far more spatially isolated or dispersal restricted (Avise 2000).

Table 3. G_{ST} values for North Atlantic eels

Species	*n*	G_{ST}	Standard error
A. rostrata	68	0.056	0.0059
A. anguilla	247	0.031	0.0045
Total	315	0.055	0.0049

Genetic Differences Between American and European Eels

Allelic frequency histograms for the two species overlap extensively (Figure 1). Thus no fixed allelic differences were observed between *A. anguilla* and *A. rostrata*, and indeed, private alleles (those present in only one species, in this case) invariably were rare in the available samples. The only partial exception involved locus *Ang101*, where one allele (*20*) was present in a frequency of 0.16 in *A. anguilla* but was not observed in *A. rostrata*. Other private alleles, albeit of low frequency, may exist in these species, but would not be detectable without vastly larger sample sizes.

An accumulation of such subtle allele frequency differences across loci probably accounts for the fact that many of the composite pairwise genetic distances between *A. anguilla* and *A. rostrata* slightly exceed those among regional populations within either species (Figure 2). Likewise, such reasoning probably explains why conspecific populations of the two recognized species tend to group separately (albeit only marginally so by bootstrap criteria) in the NJ tree (Figure 3).

This NJ tree also places the Icelandic eels in an intermediate position between those from the American and European continents. Although this genetic depiction might be interpreted as consistent with the hypothesis that some Icelandic eels are of hybrid ancestry, the available microsatellite data are inadequate to critically test this possibility. Needed instead are multilocus batteries of nuclear-gene markers that clearly distinguish *A. rostrata* from continental *A. anguilla*. No microsatellite locus assayed here remotely approaches this condition.

In principle, the paucity of allelic differentiation between American and European eels at microsatellite loci could be due to any of several phenomena. First, substantial gene flow between *A. rostrata* and *A. anguilla* would cause their allelic profiles to coincide. However, such genetic exchange seems highly unlikely because these two taxa are distinct in mtDNA composition and nearly so at the nuclear *Mdh* locus (Avise et al. 1986; Williams and Koehn 1984). Second, the extensive overlap in microsatellite allelic profiles could be due to the retention of ancestral alleles in both sister species. However, this too seems improbable given the small effective evolutionary population sizes estimated for these species (Avise et al. 1986, 1988) and their clear genetic differences at mitochondrial and at least some other nuclear loci (Avise 2003).

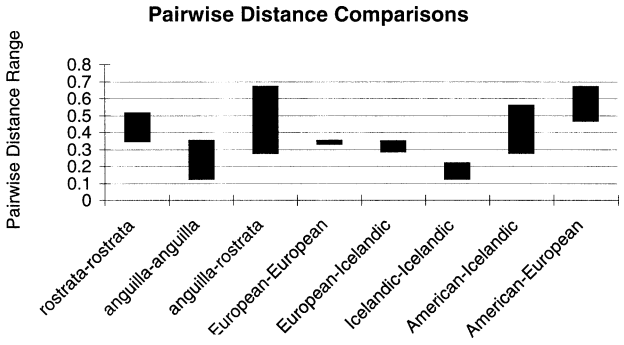

Figure 2. Range of all pairwise D_a distances (Nei et al. 1983) in comparisons between various sets of eel populations. *A. anguilla* includes samples from Europe and Iceland.

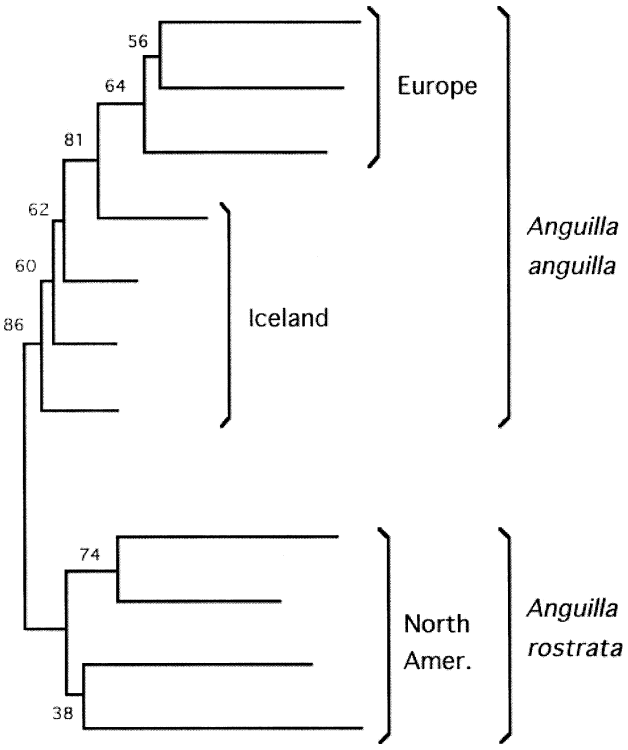

Figure 3. Neighbor-joining tree based on D_a distances, midpoint-rooted, summarizing genetic relationships of 11 geographic population samples of North Atlantic eels. Values at the nodes represent levels of bootstrap support (in percent).

Homoplasy as Noise

We hypothesize that a primary contributor to the microsatellite allelic overlap between these two species is extensive homoplasy (evolutionary convergence) associated with mutation-driven saturation effects. High microsatellite mutation rates (typically 10^{-3} in many taxa; Goldstein and Schlotterer 1999) and the stepwise nature of mismatch repair mutations (Ohta and Kimura 1973), coupled with the likelihood of physical or selective constraints at the upper end of the spectrum of allelic sizes and physical constraints at the lower end (Goldstein and Schlötterer 1999), imply that mutations to some allelic states probably occur independently and routinely in different populations (Taylor et al. 1999). If so, similar allelic frequency profiles (such as those in Figure 1) may not evidence either extensive recent genetic exchange or the retention of ancestral polymorphisms. Instead, they probably reflect, at least in part, homoplasy with regard to allelic size. This suggestion also raises a cautionary note that could apply to microsatellite allelic profiles that overlap among geographic populations within a species.

Thus, at microsatellite (or any other) loci with high mutation rates and numbers of qualitative allelic states constrained within narrow limits, many mutational alterations will merely shuffle alleles among the finite number of available slots between the boundaries. Such cryptic homoplasy can lead to significant underestimates of genetic divergence (Chapman et al. 1979; Taylor et al. 1999). The probability of homoplasy increases with divergence time (Estoup and Cornuet 1999), all else being equal, but the phenomenon also may be pervasive in large populations or those that have expanded historically. Furthermore, for populations or species that have gone through periodic or recent bottlenecks, the surviving alleles might be diagnostic for the populations in question, but nonetheless largely uninformative or even misinformative with regard to exact historical relationships.

Previous studies have identified homoplasy as an important factor contributing to overlaps in microsatellite allele frequencies among species. Judging from the long period of reproductive isolation between chimpanzees and humans, and the high mutation rates at microsatellite loci, Garza and Freimer (1996) deduced that many of the microsatellite alleles shared by these two primate species must not be identical by descent, but rather are the products of convergent evolution to common allelic states. In several species of Lake Malawi cichlids that began diverging about 700,000 years ago, detailed molecular-genetic appraisals of microsatellite alleles provided more direct evidence that homoplasy with respect to the number of repeat units was common (van Oppen et al. 2000; Zardoya et al. 1996).

At the intraspecific level also, cryptic homoplasy with regard to allelic size at the microsatellite can compromise historical analyses. Convergent evolution of this sort has been documented convincingly in, for example, horseshoe crabs (Orti et al. 1997), European rabbits (Queney et al. 2001), and humans (Garza and Freimer 1996). Such studies illustrate a growing realization that rapidly evolving loci with constrained numbers of alleles will not always be ideal for microevolutionary questions regarding historical population structure, gene flow, or the diagnosis of sibling species (Garza et al. 1995; Nauta and Weissing 1996).

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